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Paenibacillus filicis sp. nov., Isolated from the Rhizosphere of the Fern

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A Gram-positive and endospore-forming bacterial strain, designated S4^T, was isolated from the rhizosphere of ferns in Daejeon, Republic of Korea. This isolate is strictly aerobic, motile, and rod-like in shape, and it is positive for catalase, oxidase, esterase lipase, and β-galactosidase activities. In addition, this strain grows when cultured at temperatures between 15 and 37°C and at pH values ranging from 5.5 to 9.0. The DNA G+C content was determined to be 53.2 mol%. Strain S4^T has *meso*-diaminopimelic acid in the cell-wall peptidoglycan; it also contains menaquinone 7 (MK-7) as the predominant isoprenoid quinone and anteiso-$C_{15:0}$ (57.5%), iso-$C_{16:0}$ (11.3%), and $C_{16:0}$ (9.4%) as the major cellular fatty acids. Phylogenetic analysis based on alignments of the 16S rRNA gene sequence showed that S4^T is affiliated with a cluster of strains within the genus *Paenibacillaceae* and is most closely related to *Paenibacillus chinjuensis* WN9^T, with 96.8% similarity. Based on the phylogenetic and phenotypic characteristics of strain S4^T, we believe that this isolate should be distinguished from all type species of the genus *Paenibacillus* and should thus represent a novel taxon within the genus *Paenibacillus*. We propose naming this type species *Paenibacillus filicis* sp. nov. for the rhizosphere isolate; the type strain will be known as S4^T (=KCTC 13693^T =KACC 14197^T =JCM 16417^T).

Keywords: novel bacterium, fern, rhizosphere, *Paenibacillus filicis*

The genus *Paenibacillus* was originally proposed by Ash *et al*. (1993) for rRNA group 3 bacilli, a phyletic line comprising *Bacillus polymyxa* and its close relatives, on the basis of phylogenetic analysis of 16S rRNA gene sequences. Members of the genus *Paenibacillus* are strictly aerobic or facultatively anaerobic rod-shaped bacteria having ellipsoidal spores in swollen sporangia (Ash *et al*., 1993). This genus contained 105 species (http://www.bacterio.net/) (Euzeby, 1997) at the time that this manuscript was drafted, and the type species is known as *Paenibacillus polymyxa*.

Recently, a number of novel bacterial strains were isolated from the rhizosphere samples of the fern *Pteridium aquilinum* during a study to characterize culturable bacteria from the rhizosphere. One of these isolates, designated as strain S4^T, was classified as a member of the cluster comprising the *Paenibacillus* species, but it was not closely related to known Paenibacilli on the basis of 16S rRNA gene sequence comparisons. Therefore, the present study was conducted to determine the taxonomic position of this new species based on the results of polyphasic analyses, after which a new species of the genus *Paenibacillus*, herein referred to as *Paenibacillus filicis* sp. nov., is proposed.

Materials and Methods

Bacterial strains

Rhizosphere samples of ferns were collected from Mt. Geyjok in Daejeon, Republic of Korea (36° 22' 46.1" N 127° 26' 56.2" E). The samples were diluted serially and then plated onto R2A agar plates (BBL, USA) to isolate the culturable heterotrophic bacteria. The plates were incubated at 25°C for a period of 6 days. Single colonies were selected and transferred onto new R2A agar plates three consecutive times to obtain pure cultures. Strain S4^T was one of the strains isolated from the R2A agar plates. Strain S4^T was routinely cultured on R2A agar plates and stored as a glycerol suspension (20%, w/v) at -70°C. This isolate was deposited into the Korean Collection for Type Cultures (KCTC) as KACC 13693^T, the Korean Agricultural Culture Collection (KACC) as KACC 14197^T and the Japan Collection of Microorganisms (JCM) as JCM 16417^T. *P. chinjuensis* KACC 12279^T, *Paenibacillus validus* KCTC 3401^T, *Paenibacillus elgii* KCTC 10016BP^T, and *Paenibacillus soli* KCTC 13010^T were acquired from the KCTC or KACC for comparison of physiological characters.

Morphology and physiological characteristics

Gram staining was performed using a Gram stain kit (BBL). The morphology of colonies was observed with cultures on nutrient agar (NA; BBL), R2A agar, and trypticase soy agar (TSA; BBL) for 4 days at 25°C. Growth under anaerobic condition was evaluated by culturing the organism on TSA in a sealed container that contained a BBL GasPak Pouch (BD, USA). The morphology of live cells and spores was observed using light microscopy (Nikon E600; Nikon, Japan) and transmission electron microscopy (TEM, H-7600 transmission electron microscope; Hitachi, Japan) with cells that were grown for 2 days at 25°C. The physiological char-

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acteristics of the isolate were determined using cells cultivated aerobically in tryptic soy broth (TSB; BBL) or cultured on TSA for 2 days at 25°C. The motility was tested by culturing the organism in TSB medium that contained 0.4% agar. The presence of oxidase activity was detected using N,N,N9,N9-tetramethyl-1,4-phenylenediamine dihydrochloride, and the catalase activity of the strain was determined by placing drops of 3% (v/v) H_2O_2 on cultures growing on TSA and observing the cultures for the production of oxygen bubbles. To determine the optimum temperature for growth, the cells were cultured in TSA medium at temperatures ranging from 4 to 45°C for 6 days. To determine the tolerance to NaCl, cells were cultured in TSB medium in the presence of NaCl ranging from 1 to 5% (w/v) for 6 days. To determine the pH range for growth, cells were inoculated in pH-adjusted TSB media (pH 4.0 to 10.0 in 0.5 pH unit increments) at 25°C for 6 days. The growth of cells in broth culture was evaluated by measuring the optical density at 600 nm using a DU 730 UV/Vis Scanning Spectrophotometer (Beckman Coulter, USA). The growth on MacConkey agar was tested using a MacConkey agar plate (BBL). The hydrolysis of carboxymethyl-cellulose was analyzed as described previously by Rivas *et al*. (2003), the hydrolysis of casein and starch were measured using standard microbiological methods (Atlas, 1993), and the hydrolysis of Tween 80 was measured using the method described by Chakrabarty *et al*. (1970). For pectinase activity, the isolate was cultured on an R2A plate containing 0.3% citric pectin, after which the plate was stained with a solution of 1% *n*-hexadecyltrimethylammonium bromide. Additional enzyme activities of the isolate were measured using API ZYM test strips (bioMérieux, France) after an 8 h period of incubation at 30°C. An API 50CH kit and 50CHB medium (bioMérieux) were used to confirm substrate oxidation over a 2 day period at 30°C. Other biochemical and physiological characteristics of the isolate were analyzed using API 20NE and API 20E test strips (bioMérieux) over a 2 day period at 30°C.

Peptidoglycan, isoprenoid quinones, and fatty acids

The diamino acid of the peptidoglycan was determined by TLC (DC-Alufoline cellulose; Merck) as described by Komagata and Suzuki (1987). The isoprenoid quinone fraction was extracted according to the method described by Collins and Jones (1981) and was then purified via preparative TLC (silica gel F254; Merck). The identity of the quinone was determined using an HPLC (Hitachi L-5000) equipped with a reverse-phase column (YMC pack ODS-AM; YMC Co.),

Table 1. Phenotypic characteristics of strain $S4^T$ and closely related *Paenibacillus* strains
Species: 1, strain $S4^T$; 2, *P. chinjuensis* KACC 12279^T; 3, *P. validus* KCTC 3401^T; 4, *P. elgii* KCTC $10016BP^T$; and 5, *P. soli* KCTC 13010^T. Data are from this study and from Yoon *et al*. (2002), Heyndrickx *et al*. (1995), Kim *et al*. (2004), and Park *et al*. (2007). All of the strains are positive for catalase and the hydrolysis of starch. +, positive; -, negative.

Characteristic	1	2	3	4	5
Anaerobic growth	-	+	-	+	-
Motility	+	+	+	+	-
Oxidase	+	+	-	-	-
Hydrolysis of[a] :					
Casein	-	+	-	-	-
CM-Cellulose	-	-	-	+	-
Pectin	-	-	-	-	+
API test[a] :					
Acetoin production	+	+	+	-	+
Gelatinase	-	+	-	+	-
Acid production from :					
N-Acetyl-glucosamine	+	-	-	-	-
D-Cellobiose	+	-	-	-	+
Esculin	+	-	+	-	+
Galactose	+	-	-	-	-
Gentiobiose	+	-	-	-	+
Mannose	+	-	-	-	-
Mannitol	+	-	-	-	-
Maltose	+	+	+	-	+
Melibiose	+	-	-	-	-
Ribose	-	-	+	-	-
Sucrose	+	+	+	-	-
Trehalose	+	+	+	-	-
D-Turanose	+	-	+	-	-
D-Xylose	+	-	+	-	-
Growth at 40°C	-	+	+	+	+
G+C content (mol%)	53.2	53	50-52	51.7	56.6

[a] data from this study

as described by Shin *et al*. (1996). The cellular fatty acid content of strain S4^T and of closely related *Paenibacillus* strains was determined using cells that were grown on TSA agar for 2 days at 30°C. The fatty acids were extracted according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990) and were subsequently analyzed using a gas chromatograph (model 6890N and autosampler 7683; Agilent, USA) and the Microbial Identification Sherlock software package (MIDI, USA).

Determination of the G+C content and the 16S rRNA gene sequence

The genomic DNA of the isolate was extracted according to the method described by Sambrook and Russell (2001). The DNA G+C mol% was measured using an HPLC method described by Tamaoka and Komagata (1984). Briefly, the genomic DNA was hydrolyzed with nuclease P1 and dephosphorylated with alkaline phosphatase. Subsequently, the mixture of nucleosides was analyzed using an HPLC equipped with a reverse-phase column (Supelcosil LC-18-S; Supelco, Germany). The 16S rRNA gene of the isolate was amplified by PCR using the universal primers fD1 and rD1, as previously described by Weisburg *et al*. (1991). The full sequence of the 16S rRNA gene was compiled using SeqMan software (DNASTAR).

Phylogenetic analysis of the 16S rDNA

The 16S rRNA gene sequence of the isolate was compared with the available 16S rRNA gene sequences from GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/), the Ribosomal Database Project (Cole *et al.*, 2003), and the Eztaxon server (Chun *et al*., 2007) to determine its approximate phylogenetic affiliation. The 16S rRNA gene sequences of strain S4^T and of closely related type strains were aligned using CLUSTAL X software (Thompson *et al*., 1997). Genetic distances were calculated by Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were constructed using the neighbor-joining, maximum-likelihood, and maximum-parsimony methods implemented in the PHYLIP package (Felsenstein, 1993). The topologies of the phylogenetic tree were evaluated using a bootstrap analysis (Felsenstein, 1985) based on 1000 replications.

Results and Discussion

Morphology and physiological characteristics

Colonies of the strain S4^T were characterized as circular, smooth, cream in color, convex in elevation, and entire in margin on NA and TSA agar, while they were circular, rough, white in color, umbonate in elevation, and entire in margin on R2A agar, after 4 days of incubation at 25°C. Single cells of the strain S4^T were observed to be rod-like

Table 2. Cellular fatty acid profiles of strain S4^T and closely related *Paenibacillus* species
Species: 1, strain S4^T; 2, *P. chinjuensis* KACC 12279^T; 3, *P. validus* KCTC 3401^T; 4, *P. elgii* KCTC 10016BP^T; and 5, *P. soli* KCTC 13010^T. The values represent the percentages of the total fatty acid content. Fatty acids comprising less than 0.5% of the total in all strains were excluded. All strains were cultured on TSA for 2 days at 30°C. -, not detected.

Fatty acid	1	2	3	4	5
Saturated					
$C_{10:0}$	0.6	-	0.2	-	0.3
$C_{12:0}$ 3OH	-	1.7	-	-	-
$C_{13:0}$ 2OH	-	5.4	-	-	-
$C_{14:0}$	1.9	0.7	1.3	1.3	1.7
$C_{15:0}$ 2OH	-	0.5	-	-	-
$C_{16:0}$	9.4	7.0	8.8	4.9	8.2
$C_{17:0}$	0.5	0.5	0.2	-	0.5
Unsaturated					
$C_{16:1}\omega 7c$ OH	-	-	1.7	0.7	-
$C_{16:1}\omega 11c$	-	-	3.8	3.5	-
iso $C_{17:1}\omega 10c$	-	-	1.0	0.7	-
Branched					
iso-$C_{12:0}$ 3OH	-	0.8	-	-	-
iso-$C_{13:0}$ 3OH	-	0.7	-	-	-
iso-$C_{14:0}$	4.4	1.3	2.2	2.1	2.7
iso-$C_{15:0}$	4.8	1.5	11.2	21.2	12.4
iso-$C_{16:0}$	11.3	7.1	8.3	4.8	6.5
iso-$C_{17:0}$	2.3	0.9	5.0	3.1	1.8
anteiso-$C_{15:0}$	57.5	63.1	48.7	53.3	60.3
anteiso-$C_{17:0}$	5.2	8.3	6.5	4.5	2.6
Summed features[a]					
1	-	0.6	-	-	-
3	-	-	-	-	1.5

[a] Summed features represent groups of two or three fatty acids that could not be separated using GLC with the Microbial Identification System (MIDI). Summed feature 1 contains one or more of the following fatty acids: $C_{13:0}$ 3OH and/or iso-$C_{15:1}$ H. Summed feature 3 contains one or more of the following fatty acids: $C_{16:1}\omega 7c$ and/or $\omega 6c$.

in shape, 0.7 to 0.9 μm in width, and 2.2 to 2.9 μm in length. The terminal ellipsoidal spore was observed in the swollen sporangia of the strain. In addition, strain $S4^T$ was strictly aerobic, motile, oxidase-positive, catalase-positive, and Gram-positive. The strain was also capable of growth on TSB medium that contained 0% to 3% (w/v) NaCl. However, it did not grow in the presence of ≥4% (w/v) NaCl. Growth of strain $S4^T$ was observed at temperatures between 15 and 37°C, although the optimal temperature range was between 25 and 30°C. No signs of growth were observed below 10°C or above 40°C. The initial pH range for which growth of strain $S4^T$ was observed was between pH 5.5 and 9.0; however, the optimal pH value for growth was determined to be 7.5. The isolate was not able to grow on MacConkey agar. For comparative studies, the physiological characteristics of closely related type strains were tested together with that of strain $S4^T$. The physiological properties of strain $S4^T$ and the related type strains are shown in Table 1.

Peptidoglycan, isoprenoid quinones, and fatty acids

Strain $S4^T$ contained *meso*-diaminopimelic acid in its cell-wall peptidoglycan. The predominant isoprenoid quinone was MK-7, and the principal cellular fatty acids of cells grown on TSB agar for two days were anteiso-$C_{15:0}$ (57.5%), iso-$C_{16:0}$ (11.3%), and $C_{16:0}$ (9.4%). The fatty acid profiles representing the differences among strain $S4^T$ and related type strains are shown in Table 2. It has previously been reported that the *Paenibacillus* strains express MK-7 as the major respiratory quinone and anteiso-$C_{15:0}$ as the major fatty acid (Shida *et al*., 1997).

G+C content and phylogenetic analysis

The DNA G+C content of strain $S4^T$ was determined to be 53.2 mol%. The sequence of its 16S rRNA gene was aligned with representative strains of the genus *Paenibacillus* and

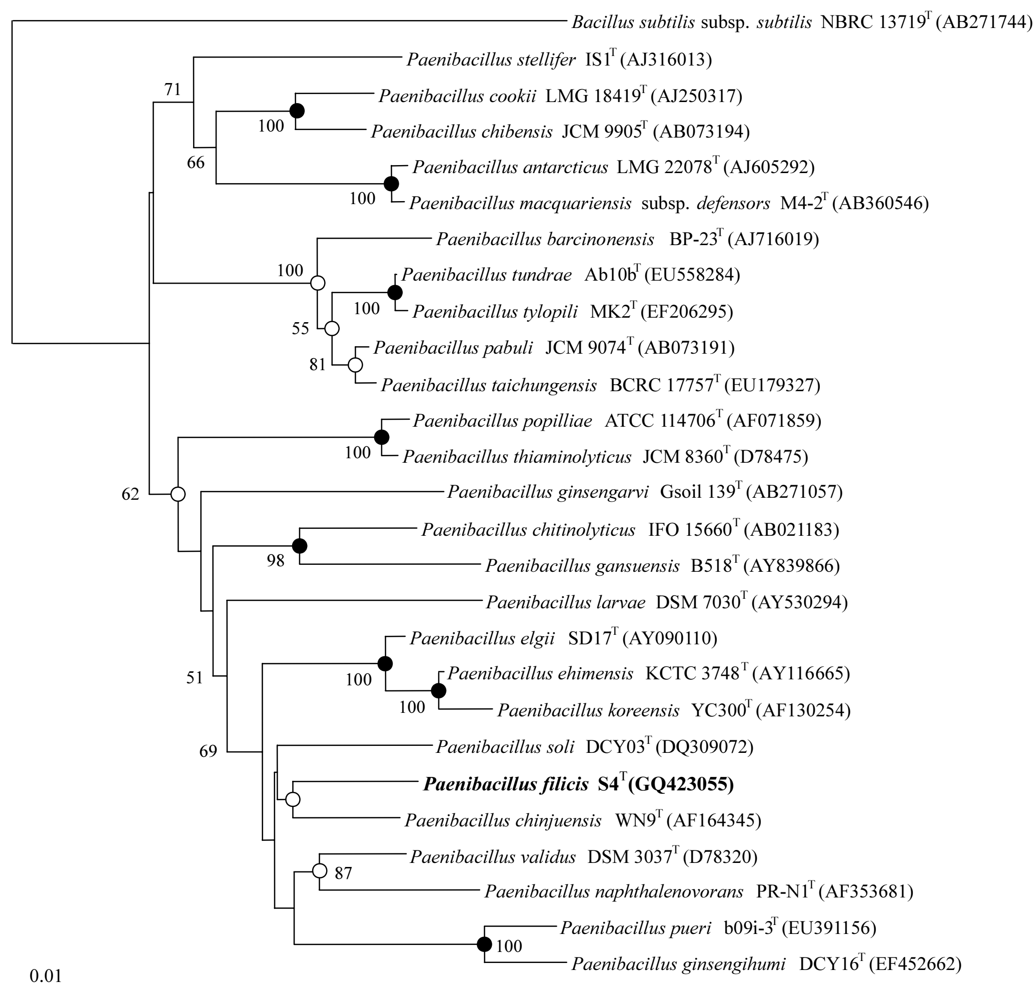

Fig. 1. Phylogenetic dendrogram of strain $S4^T$ and related strains in the genus *Paenibacillus* based on 16S rRNA gene sequence similarity. The tree was constructed using the neighbor-joining method. Closed circles indicate that the corresponding nodes are also recovered in both maximum-likelihood and maximum-parsimony methods. Open circles indicate that the nodes are recovered either in the maximum-likelihood or maximum-parsimony method. Bootstrap values (1,000 replications) are shown as percentages at each node only if they are 50% or greater. The GenBank accession no. of the 16S rRNA gene sequences are provided in parentheses. *Bacillus subtilis* subsp. *subtilis* NBRC 13719^T was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

other related taxa. We found that strain S4^T was between 90.2 and 96.8% similar to the 16S rRNA gene of all of the *Paenibacillus* species type strains. The 16S rRNA gene sequence of strain S4^T showed the highest similarity with that of *P. chinjuensis* WN9^T (96.8%), with the next highest similarities being with *P. validus* JCM 9077^T (96.7%) and *P. elgii* SD17^T (96.4%). The phylogenetic tree showing the relationship between the isolate and representatives of the genus *Paenibacillus* is shown in Fig. 1. In the trees constructed with the neighbor-joining, maximum-likelihood, and maximum-parsimony methods, bootstrap support for the trees was low. However, strain S4^T was contained in a similar group presented in all the three trees. It should be noted that the strain S4^T was genetically distinct from the type species of *Paenibacillus*. According to the work of Wayne *et al*. (1987) and Stackebrandt and Goebel (1994), strains that have less than 97.0% 16S rRNA gene sequence homology can be considered as different species. Thus, the results of these phylogenetic analyses demonstrated that strain S4^T was not related to any of the recognized members of the genus *Paenibacillus* at the species level. The partial 16S rDNA gene sequence of S4^T, which was 1,467 bp in length, has been deposited in the GenBank nucleotide database under the accession number GQ423055 at the NCBI website (http://www.ncbi.nlm.nih.gov).

On the basis of the data presented herein, we propose the identification of a novel species from the genus *Paenibacillus*, referred to as *Paenibacillus filicis*, which was an organism isolated from the rhizosphere of a fern.

Description of *Paenibacillus filicis* sp. nov.

Paenibacillus filicis (fi'li.cis: L. gen. n. *filicis*, of a fern plant) The cells are Gram-positive, strictly aerobic, catalase-positive, oxidase-positive, motile, and have a terminal ellipsoidal spore localized in the swollen sporangia. The colonies grown on TSA agar are characterized as circular, smooth, cream, convex, and entire margin. The diameters of the colonies that grew on NA, R2A, and TSA agar are 1.5, 2.5, and 3.0 mm, respectively, after an incubation period of 4 days at 25°C. The single cells are rod-shaped and measured 0.7 to 0.9×2.2 to 2.9 μm. Growth occurs at temperatures between 15 and 37°C in TSB medium, with the optimum growth rate occurring between 25 and 30°C, at pH values ranging from 5.5 to 9.0, and in the presence of 0 to 3% NaCl. Hydrolyze starch and Tween 80 but not casein, CM-cellulose, and pectin. In API 20E and 20NE, it assimilates N-acetyl-glucosamine, d-glucose, d-maltose, d-mannitol, and d-mannose but does not assimilate adipate, l-arabinose, caprate, citrate, gluconate, malate, or phenyl-acetate. In addition, a negative reaction is observed for arginine dihydrolase, gelatinase, H_2S production, indole production, lysine decarboxylase, nitrate reduction, tryptophane deaminase, and urease. In the API ZYM test, the reaction is positive for N-acetyl-β-glucosaminidase, esterase lipase, β-glucosidase, and leucine arylamidase activities, but it is negative for acid phosphatase, α-chymotrypsin, crystine arylamidase, esterase, α-fucosidase, α-galactosidase, β-glucuronidase, lipase, α-mannosidase, naphtol-AS-BI-phosphohydrolase, and valine arylamidasetrypsin activities. In the API CH50 reaction, an acid is produced from N-acethyl-glucosamine, d-cellobiose, d-galactose, gentiobiose, d-glucose, glycogen, d-maltose, d-mannose, d-mannitol, d-melibiose, d-raffinose, sucrose, d-trehalose, d-turanose, and d-xylose but not from adonitol, d-arabitol, l-arabitol, d-arabinose, l-arabinose, dulcitol, erythritol, d-fucose, l-fucose, 2-keto-gluconate, 5-keto-gentiobiose, gluconate, inositol, inulin, d-lyxose, α-methyl-d-mannopyranside, α-methyl-d-glucoside, d-melezitose, d-ribose, l-rhamnose, d-sorbitol, l-sorbose, d-tagatose, l-xylose, or xylitol. Cells cultured in TSB medium contain anteiso-$C_{15:0}$ as the predominant cellular fatty acid. In addition, the predominant isoprenoid quinone is MK-7. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The G+C content of the type strain is 53.2 mol%. The type strain is S4^T (=KCTC 13693^T =KACC 14197^T =JCM 16417^T).

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